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MUTABILITY OF INFLUENZA VIRUS AFTER TREATMENT WITH NITROUS ACID

[Following is the translation of an article by N. P. Obrosova-Serova and M. I. Sokolov, Institute of Virology imeni D. I. Ivanovskogo, AMN USSR, Moscow, published in the Russian-language periodical Voprosy Virusologii (Problems of Virology), No 6, 1966, pages 658-662. It was submitted on 8 Feb 1965.]

In recent years the problem of chemical mutagenesis in viruses has become the subject of experimental investigation [2, 4, 10, 11, 14, 15]. The most detailed studies have been made of mutants obtained by treatment with nitrous acid on the viruses of tobacco mosaic (Gierer, Mundry [7], Siege [11]), poliomyelitis (Boeye [5], Carp, Koprowski [6], Yu. Z. Gendon [1]), the ECHO 9 virus (Goldblum, Margalith, M., Margalith, E. [8]), the virus of Newcastle disease (Granoff [9], Thiry [13]), and phage (Tessman [12], Freese, Freese [4]; A. S. Kriviskiy, [3], A. Lysenko [3]).

In the present report data are presented on the obtaining of mutants of the influenza A2 virus under the action of nitrous acid.

Materials and Methods

The investigations were conducted on the selectively-grown FM33 virus of influenza type A2 which is inhibitor-resistant and pathogenic for white mice.

As the mutagen we used a 4 M solution of nitrous acid. The virus was treated in the following manner: 2 parts of undiluted allantoic culture of virus were mixed with 1 part of acetate buffer (pH 4.7) and 1 part of a 4 M solution of nitrous acid. After a specific time of contact between the virus and nitrous acid (at 20°) samples were taken which were diluted 10-fold with a 1 M phosphate buffer (pH 7.8) for stopping the action of the mutagen. Then 0.1 ml of processed virus was introduced into the allantoic cavity of 10-11 day old chick embryos.

Pathogenicity for mice, infectious titer for chick embryos, and hemagglutinating activity of the virus were determined by the usually accepted methods. Sensitivity of the viruses to inhibitors was established in the hemagglutination inhibition reaction (HIR) with normal horse serum which was heated at 62° for 45 minutes. Antigenic and immunogenic properties of strains were determined on mice which

were immunized subcutaneously one time with 0.5 ml of undiluted virus. Fermentation activity was calculated by the rate of elution of virus from chick erythrocytes at 37°. Heat resistance of hemagglutinins and infectious virus were established by means of heating of virus in a water bath at 56°.

Results

In preliminary tests we studied the sensitivity of the strain to the inactivating action of nitrous acid. As can be seen from table 1, the inactivating action of nitrous acid was manifested on virus in 8 minutes of contact.

Table 1

Results of the inactivation of influenza A2 virus (strain FM_{33}) by nitrous acid

(a) Время контакта вируса с азотистой кислотой (в мин.)	(b) Наличие вируса в куриных эмбрионах, зараженных вирусом, обработанным азотистой кислотой	
	(c) число куриных эмбрионов, положительных в РГА	(d) число зараженных куриных эмбрионов
7	8	8
8	6	8
9	4	8
10	2	8
11	3	8
13	1	8
14	2	8

Key: (a) Time of contact of virus with nitrous acid (in minutes); (b) Presence of virus in chick embryos which were infected with virus which had been treated with nitrous acid; (c) number of chick embryos, positive in the HR; (d) number of infected chick embryos.

However, as it was cleared up, allantoic fluids which were negative in the HR still contained viable virus, which was revealed in the 2nd passage of allantoic fluids which were negative in the HR (Table 2).

In the allantoic fluid of embryos, infected with material which was treated with nitrous acid for 14 minutes, virus was not revealed in the passages.

A study was made of virus which was isolated after the 2nd passage of HR negative material which was treated with nitrous acid for 13 min. This culture possessed a higher hemagglutinating activity with chick erythrocytes in comparison with the initial strain. Already in the 1st passage in chick embryos by the method of end dilutions a heterogeneity was revealed in the virus population: along with clones which agglutinated erythrocytes in large dilutions (1:2560), there were cultures which caused agglutination in low dilutions (1:10). In the

course of 3 subsequent passages of end dilutions of allantoic fluids with high ("high" line) and low ("low" line) titers of hemagglutinins they were homogeneous based on this feature. In each line a study was made of 2 clones which were isolated after 4 passages of end dilutions of virus.

Table 2

Results of the isolation of virus from allantoic fluids, which were negative in the HR, after the 2nd passage in chick embryos

(a) Время контакта с азотистой кислотой (в мин.)	(b) Титры гемагглютининов в куриных эмбрионах, зараженных негативными по РГА аллантоисными жидкостями				
	1	2	3	4	5
10	1: 640	1:1 280	1:2 560	—	—
11	1: 320	—	—	—	—
12	1: 320	1:1 280	1: 640	1:2 560	1:320
13	1:1 280	1:5 120	1:1 280	—	—
14	—	—	—	—	—

Legend: — - HR negative; 1—5 - serial number of embryos.

Key: (a) Time of contact with nitrous acid (in min); (b) Titers of hemagglutinins in chick embryos, infected with allantoic fluids which were negative in the HR.

Simultaneously with this we studied the biological properties of 2 clones which were isolated from the initial strain (not subjected to the influence of nitrous acid) by means of 3 subsequent passages of end dilutions of virus. For each subsequent passage we selected the cultures with the lowest titer of hemagglutinins. Selection of the initial strain showed that the population of virus was homogeneous and did not contain virus particles with a low titer of hemagglutinins. The clones possessed properties which were characteristic for the initial strain. In table 3 the results are cited from a study of the properties of the isolated mutants.

As can be seen from table 3, mutants of the "low" line (6 ⋗ 14 and 7 ⋗ 13) lost their pathogenicity and toxicity for white mice and possessed a lower infecting capacity for chick embryos and a very weak immunogenic activity. Together with this they acquired a sensitivity to inhibitors of normal horse serum and eluted from erythrocytes more slowly than the initial strain. A study of the resistance of hemagglutinins to heating at 56° showed that the hemagglutinating activity of mutants was preserved for 90 minutes of heating, and the initial strain and its clones were inactivated in 30 minutes. Along with this the mutants of the "low" line preserved the ability to agglutinate mice erythrocytes.

Table 3

Properties of mutants, obtained under the influence of nitrous acid

(a) Штамм	(b) Титр гемагглютининов с эритроцитами		(e) Чувствительность к ингибитору	(f) ID_{50} (в lg)	(g) LD_{50} (в lg)	(h) Токсичность	(i) Активность элюции с эритроцитов кур	(j) Термоустойчивость гемагглютининов (в мин.)	(k) Иммуногенность (резистентность к LD_{50})
	(c) кур	(d) мышей							
(l) $ФМ_{33}$ (исходный)	1:1 280	1:640	P	8,0	5,0	+	B	30	10 000
(m) Клоны ФМ 7э9[1]	1:640	1:160	»	7,0	4,0	+	»	30	. . .
8э23	1:640	1:160	»	7,0	5,0	+	»	30	. . .
(n) Мутанты „низкой" линии 6э14	1:10	1:4	1:5 120	5,5	—	—	H	90	1
7э13	1:20	1:4	1:5 120	6,5	—	—	»	90	<1
(o) Клоны „высокой" линии 9э3	1:2 560	1:640	P	8,0	5,5	+	H	30	10 000
9э4	1:1 280	1:640	»	7,5	5,0	+	»	30	>1 000

Legend: Here and in table 4: + presence of feature; — absence of feature; . . . . . investigations not conducted.

P - resistance to inhibitor of normal horse serum; B - high eluting activity; H - low eluting activity.

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[1] - 7 - negative logarithm of dilution of virus from which the studied variant was taken; Э14 - number of embryo.

Key: (a) Strain; (b) Titer of hemagglutinins with erythrocytes; (c) chick; (d) mice; (e) Sensitivity to inhibitor; (f) ID_{50} (in lg); (g) LD_{50} (in lg); (h) Toxicity; (i) Activity of elution from erythrocytes of chickens; (j) Thermoresistance of hemagglutinins (in min.); (k) Immunogenicity (resistance to LD_{50}); (l) FM_{33} (initial); (m) Clones of FM 7Э9[1] and 8Э23; (n) Mutants of "low" line 6Э14 and 7Э13; (o) Clones of "high" line 9Э3 and 9Э4.

Clones of the "high" line (-9э3 and -9э4) possessed properties which were inherent to the initial strain, with the exception of the slower elution from chick erythrocytes (see drawing). The initial strain (-7э9) eluted from chick erythrocytes in 30 minutes, and clones -9э3 and -9э4 - in 2 hours.

Further we checked the stability of the isolated mutants by means of passaging them in chick embryos and in mice (table 4).

It can be seen from table 4 that passages of mutants of the "low" line in chick embryos led to an increase in the titers of hemagglutinins with erythrocytes of chicks and mice, and also to a certain

amplification in the activity of multiplication in the allantoic cavity of chick embryos. No other changes were revealed in mutants -6э14 and -7э13.

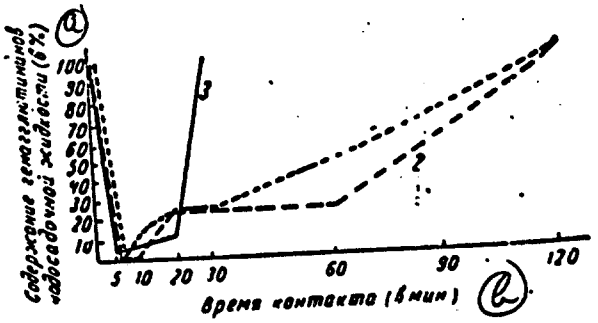
Table 4

Properties of mutants after passages in chick embryos and in mice

Клоны штамма $ФМ_{33}$	Число пассажей	Титр гемагглютининов с эритроцитами кур	Титр гемагглютининов с эритроцитами мышей	Чувствительность к ингибитору	ID_{50} (в lg)	LD_{50} (в lg)	Токсичность	Активность элюции с эритроцитов кур	Термоустойчивость гемагглютининов (в мин.)
6э14	5 в куриных эмбрионах	1:160	1:40	10 240	7,0	—	—	Н	>120
7э13		1:160	1:40	10 240	7,0	—	—	»	>120
9э3		1:1280	1:320	Р	7,5	4,0	+	В	30
9э4		1:1280	1:640	«	7,5	4,0	+	»	30
6э14	5 на мышах	1:320	1:320	Р	. . .	1,0	—	. . .	. . .
7э13		1:40	1:40	160 10 240	. . .	—	—	. . .	. . .

Key: (a) Clones of strain FM_{33}; (b) Number of passages; (c) Titer of hemagglutinins with erythrocytes of; (d) chicks; (e) mice; (f) Sensitivity to inhibitor; (g)(In lg); (h) Toxicity; (i) Activity of elution from chick erythrocytes; (j) Thermoresistance of hemagglutinins (in min.); (k) 5 in chick embryos; (l) 5 on mice.

Legend same as in table 3.



Activity of elution from erythrocytes of mutants of the "high" line. 1 - mutant 9э3; 2 - mutant 9э4; 3 - 7э9 strain FM_{33}.

Key: (a) Contents of hemagglutinins of supernatant fluid (in %); (b) Time of contact (in minutes)/ .

After passage in chick embryos clones of the "high" line recovered the ability to actively elute from chick erythrocytes.

We also studied certain properties of mutants of the "low" line

after 5 passages through the lungs of white mice. Mutant -6314 acquired weak virulence (10^{-1}), causing the death of mice in the usual periods. The results of the investigation of the virus population of this mutant for sensitivity to the inhibitor of horse serum showed that it consisted of a mixture of sensitive and resistant forms. In mutant (-7313) reversion of virulence for mice and resistance to inhibitor of normal horse serum was not observed.

Conclusions

1. Following the influence of nitrous acid on the influenza A2 virus mutants emerged which lost their pathogenic, toxic, and immunogenic properties, and also the resistance to the inhibitor of normal horse serum. In comparison with the initial strain the mutants multiplied less actively in chick embryos and eluted more slowly from chick erythrocytes.

2. Clones which caused the agglutination of chick erythrocytes in high titers were distinguished from the initial pathogenic strain by a weakly expressed eluting activity which, however, was regained after 5 passages in chick embryos.

3. When mutants of the "low" line were passaged on mice the properties were either preserved or virulence for mice increased insignificantly with a loss of sensitivity to the inhibitor.

4. Loss of virulence may be accompanied by a transition of the inhibitor-resistant form of virus to a sensitive one, and acquisition of pathogenicity is frequently connected with a loss of sensitivity to the inhibitor.

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